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14. ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal dominant multisystem disorder caused by loss of either TSC1 or TSC2 function(Tsai and Sahin, 2011). TSC affects 1/6,000 individuals worldwide and affects multiple organs including the brain, skin, eyes, kidneys, heart, and lungs(Crino et al., 2006). TSC patients present with epilepsy (~90%), intellectual disability and autism (~50%), and other disorders including sleep disruption, attention-deficit hyperactivity disorder, and anxiety(Han and Sahin, 2011). The neuropathological findings in TSC are cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs)(DiMario, 2004). Another important yet not well-studied feature of TSC pathology in brain is hypomyelination(Ridler et al., 2001). Most recently using diffusion tensor imaging we observed abnormal white matter microstructure in patients with TSC that have autism compared to TSC patients without autism (Lewis et al., 2013; Peters et al., 2013). To uncover the underlying molecular mechanisms of hypomyelination in TSC, we investigated the role of neuronal factors affecting oligodendrocyte development in our Tsc1cc;SynlCre+ mouse model, which lacks Tsc1 expression only in neurons. Here we show that, neurons lacking Tsc1 secrete excessive amounts of connective tissue growth factor (CTGF), which in turn blocks the maturation of oligodendrocytes, and thus myelination both in vitro and in vivo.

15. SUBJECT TERMS
Nothing listed

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Table of Contents

1. INTRODUCTION:

Tuberous sclerosis complex (TSC) is an autosomal dominant multisystem disorder caused by loss of either TSC1 or TSC2 function(Tsai and Sahin, 2011). TSC affects 1/6,000 individuals worldwide and affects multiple organs including the brain, skin, eyes, kidneys, heart, and lungs(Crino et al., 2006). TSC patients present with epilepsy (~90%), intellectual disability and autism (~50%), and other disorders including sleep disruption, attention-deficit hyperactivity disorder, and anxiety(Han and Sahin, 2011). The neuropathological findings in TSC are cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs)(DiMario, 2004). Another important yet not well-studied feature of TSC pathology in brain is hypomyelination(Ridler et al., 2001). Most recently using diffusion tensor imaging we observed abnormal white matter microstructure in patients with TSC that have autism compared to TSC patients without autism (Lewis et al., 2013; Peters et al., 2013). To uncover the underlying molecular mechanisms of hypomyelination in TSC, we investigated the role of neuronal factors affecting oligodendrocyte development in our *Tsc1^{cc};SynICre^{+}* mouse model, which lacks Tsc1 expression only in neurons. Here we show that, neurons lacking Tsc1 secrete excessive amounts of connective tissue growth factor (CTGF), which in turn blocks the maturation of oligodendrocytes, and thus myelination both *in vitro* and *in vivo*.

2. KEYWORDS:

Tuberous Sclerosis Complex, myelination, CTGF

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

In this report, we will summarize the progress we made throughout the whole grant period. Our major findings have been submitted as a full manuscript and are currently under review. We will review the accomplishments in each aim as approved in our SOW last year. The figures are in the appendices.

Aim 1A: To determine the role of CTGF in hypomyelination in the mouse model. Here we sought to determine the role of CTGF protein in myelination abnormalities using a TSC mouse model (*Tsc1^{cc};Syn-Cre1^{+}).*

I. We will generate two independent *CTGF*-shRNAs and confirm their efficacy in vitro.

As detailed in last year's progress report, we did not proceed with these *in vitro* experiments since the *in vivo* experiments detailed below gave extremely compelling results allowing us to focus on the role of CTGF in the mouse model directly.

II. We will generate AAV2-CTGF-shRNA to reduce the expression of CTGF in vivo. AAV2-expressing scrambled shRNA as a control to the contralateral hemisphere. Alternatively, we will generate mice missing both *Tsc1* and *Ctgf* genes under the Syn-Cre promoter (see grant proposal for details). Then we will follow look at MBP staining in the double knockout mice compared to single *Tsc1* knockout mice.

We crossed *Tsc1;SynICre*$^{+}$ mice with *CTGF*$^{f/f}$ mice (Kapoor et al., 2008) to generate the following groups of mice: wild type for both genes (*Tsc1*^{ww}*;SynICre*$^{+}$*;CTGF*$^{+/+}$), mutant for *Tsc1* and wild type for *CTGF* (*Tsc1*^{cc}*;SynICre*$^{+}$*;CTGF*$^{+/+}$) and mutant for both genes (*Tsc1*^{cc}*;SynICre*$^{+}$*;CTGF*$^{f/f}$). To our knowledge, this is the first mouse model lacking CTGF only in neurons. We stained the brain sections of control, mutant and double mutant with the MBP antibody to visualize myelination and found that loss of CTGF partially rescued the hypomyelination phenotype in Tsc1 mutant mice as assessed by the increased MBP signal (Figure 1). Furthermore, CTGF deficiency by itself in an otherwise wild-type mouse model, induced increased MBP staining, arguing that CTGF is a break on myelination under physiological conditions (Figure 2).

Aim 1B: To test whether CTGF expression is altered in human TSC brain.

We have initiated staining of CTGF in tuber specimens from TSC patients taken at the time of epilepsy surgery. We stained paraffin sections of tubers from TSC patients and detected that CTGF is expressed in the tuber. We found that all phospho-S6 positive cells are also CTGF-positive. We also asked whether CTGF levels are also elevated in iPSC-derived human neurons from TSC patients. We generated iPSC lines from fibroblasts collected from TSC patients and unaffected family members to use as controls, and differentiated these cells into neurons. Compared to unaffected controls, iPSC-derived neurons from fibroblasts of TSC patients showed elevated levels of CTGF protein.

Aim 2: To examine the mechanisms by which CTGF regulates oligodendrocyte differentiation.

Our preliminary data from our first-year report suggests that the Mod-IV, which is one of the domains of CTGF is responsible for inhibiting oligodendrocyte maturation. To address this question systematically, we have been generating CTGF expression constructs, which express different domains of CTGF (Mod-I, Mod-II, Mod-III, Mod-IV and their combinations) to test the mutual and/or complementary effects of these different domains on oligodendrocyte maturation. So far we have generated the following FLAG-tagged constructs: Full-length, Module I, Module I+II. We had some difficulty expressing all the fragments with equal efficiency. We had showed that the CM collected from HEK293T cells expressing the full-length FLAG-CTGF affects the maturation of oligodendrocytes. We have not yet determined which module is necessary and sufficient for the effect of CTGF on oligodendrocyte maturation assay.

We also initiated an investigation of how TSC-deficiency regulates CTGF expression. We focused on the role of protein serum response factor, SRF. Previous reports show that the SRF functions as the repressor of *Ctgf* transcription (Stritt et al., 2009). Importantly, a previous study showed that neurons lacking SRF have higher expression of CTGF and thus blocks the

maturation of oligodendrocytes (Stritt et al., 2009). We therefore analyzed the levels of SRF both in our mutant mice and in primary cortical neurons lacking *Tsc2*. Both SRF protein and transcript levels were decreased in *Tsc2* KD neurons compared to the control neurons (Figure 3). In addition, we checked the transcript levels of other targets of SRF, *Egr1* and *Cyr61*. SRF functions as a transcription activator of *Egr1* (Kim et al., 2008), whereas it suppresses the transcription of *Cyr61* (Stritt et al., 2009), which is in the same CCN family of proteins as CTGF (Jun and Lau, 2011). In Tsc2 KD cortical neurons, both the transcript levels of SRF and *Egr1* are decreased, whereas *Cyr61* is increased as *Ctgf*. Moreover, when stained with SRF antibody, compared to the control mice, the mutant mice brain sections showed decreased intensity, suggesting SRF expression is diminished both *in vivo* and *in vitro.* Together, our data suggest that the upregulation of CTGF in *Tsc*-deficient neurons may be due to the downregulation of it repressor, SRF.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

The sentinel finding of our research include:
(1) Loss of Tsc1 only in neurons is sufficient to reduce mature oligodendrocyte number, thus myelination in the brain.
(2) Conditioned media from Tsc-deficient neurons leads to reduction in oligodendrocyte maturation in culture.
(3) Tsc-deficient neurons overexpress CTGF.
(4) Loss of TSC1/2 reduces SRF, which is a known suppressor of CTGF expression.
(5) Double knockout of *Tsc1* and *Ctgf* in neurons leads to improved myelination.

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

Our findings suggest that the neuronal CTGF is a strong determinant of myelination in vivo. Future studies of the downstream effects of CTGF on oligodendrocytes and identification of the protein module(s) within CTGF that are responsible for regulation of oligodendrocyte development will be a major goal for the discovery of new treatment options. Our study provides the first description of a possible molecular mechanism that could underlie the aberrant white matter microstructure in TSC patients. The non cell-autonomous effect of CTGF should also be investigated in other diseases associated with myelination deficits such as periventricular leukomalacia and cerebral palsy, and as well in demyelinating diseases such as multiple sclerosis.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed

scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press: none

(2) Peer-Reviewed Scientific Journals:

Ebru Ercan, Juliette M. Han, Kellen D. Winden, Min-Joon Han, Leonie Hoyo, Alessia Di Nardo, Andrew Leask, Daniel H. Geschwind, Mustafa Sahin. Neuronal CTGF/CCN2 Regulates Oligodendrocyte Maturation and Myelination in a Mouse Model of Tuberous Sclerosis Complex (under review)

(3) Invited Articles:

Lipton JO and Sahin M. The Neurology of mTOR. Neuron. 2014 Oct 22;84(2):275-91.

Ebrahimi-Fakhari D. and Sahin M. Autism and the Synapse: Emerging Mechanisms and Mechanism-based Therapies. Curr Opin Neurol. 2015 Apr;28(2):91-102.

DiMario FJ Jr, Sahin M, Ebrahimi-Fakhari D. Tuberous Sclerosis Complex. Pediatr Clin North Am. 2015 Jun;62(3):633-648

Davis PE, Peters JM, Krueger DA and Sahin M. Tuberous Sclerosis: A new frontier in targeted treatment of autism. Neurotherapeutics 2015 Jul;12(3):572-83.

Sahin M and Sur M. Genes, Circuits, and Precision Therapies for Autism and Related Neurodevelopmental Disorders. Science 2015 Nov 20;350(6263). pii: aab3897.

(4) Abstracts:

Ebru Ercan, Juliette M. Han, Jianlin Wang, Kellen Winden, Duyu Nie, Daniel H. Geschwind, Paul Rosenberg, Mustafa Sahin. The Role of Neuronal Tuberous Sclerosis Complex in Oligodendrocyte Maturation. Embo Conference. Brain development and disorders 5 – 8 September 2014 | La Ciotat, France

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

2015 **Tuberous Sclerosis: Shedding light on the neural circuitry of autism** / Lecture
UCSF Symposium on Autism
San Francisco, CA

2015 **Dissecting the Neural Circuitry of Autism** / Seminar
Hope Center for Neurological Disorders
Washington University School of Medicine
St. Louis, MO

2015 **Targeted Treatments for Tuberous Sclerosis** / Lecture
Pathways of Neurodevelopmental Disorders / Organizer

Keystone Symposium
Tahoe City, CA

2015 **Tuberous Sclerosis: Shedding light on the neural circuitry of autism** / Lecture
University of Massachusetts Memorial Medical Center
Worcester, MA

2015 **Tuberous Sclerosis: Shedding light on the neural circuitry of autism** / Lecture
The 28th Annual Rita G. Rudel/Lucy M. Moses Lecture
Neurology Department
Columbia University Medical Center
New York, NY

2015 **Neuronal Connectivity in Tuberous Sclerosis** / Keynote
Stanley Manne Children's Research Institute
Northwestern University
Developmental Biology Program Research Symposium
Chicago, IL

2015 **Dissecting the Neural Circuitry of Autism /** Lecture
Neurogenetics Symposium
Duke University
Raleigh, NC

2015 **Translational studies in tuberous sclerosis** / Lecture
Educational Symposium
International Meeting for Autism Research
Salt Lake City, UT

2016 **Tuberous Sclerosis as a model for autism spectrum disorder /** Lecture
UCLA Center fro Autism Research and Treatment
Los Angeles, CA

2016 **Tuberous Sclerosis: Shedding light on the neural circuitry of autism /** Grand Rounds
Department of Neurology
Albert Einstein College of Medicine
Bronx, NY

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or

rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

No products that may be commercialized.

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Nothing to report

10. REFERENCES:

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Han, J.M., and Sahin, M. (2011). TSC1/TSC2 signaling in the CNS. FEBS letters 585, 973-980.

Jun, J.I., and Lau, L.F. (2011). Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nature reviews Drug discovery 10, 945-963.

Kapoor, M., Liu, S., Huh, K., Parapuram, S., Kennedy, L., and Leask, A. (2008). Connective tissue growth factor promoter activity in normal and wounded skin. Fibrogenesis & tissue repair 1, 3.

Kim, M.J., Kang, J.H., Chang, S.Y., Jang, H.J., Ryu, G.R., Ko, S.H., Jeong, I.K., Kim, M.S., and Jo, Y.H. (2008). Exendin-4 induction of Egr-1 expression in INS-1 beta-cells: interaction of SRF, not YY1, with SRE site of rat Egr-1 promoter. Journal of cellular biochemistry 104, 2261-2271.

Lewis, W.W., Sahin, M., Scherrer, B., Peters, J.M., Suarez, R.O., Vogel-Farley, V.K., Jeste, S.S., Gregas, M.C., Prabhu, S.P., Nelson, C.A., 3rd, and Warfield, S.K. (2013). Impaired language pathways in tuberous sclerosis complex patients with autism spectrum disorders. Cerebral cortex 23, 1526-1532.

Peters, J.M., Taquet, M., Prohl, A.K., Scherrer, B., van Eeghen, A.M., Prabhu, S.P., Sahin, M., and Warfield, S.K. (2013). Diffusion tensor imaging and related techniques in tuberous sclerosis complex: review and future directions. Future neurology 8, 583-597.

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Stritt, C., Stern, S., Harting, K., Manke, T., Sinske, D., Schwarz, H., Vingron, M., Nordheim, A., and Knoll, B. (2009). Paracrine control of oligodendrocyte differentiation by SRF-directed neuronal gene expression. Nature neuroscience 12, 418-427.

Tsai, P., and Sahin, M. (2011). Mechanisms of neurocognitive dysfunction and therapeutic considerations in tuberous sclerosis complex. Current opinion in neurology 24, 106-113.

11. APPENDICES: N/A

NOTE:

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COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

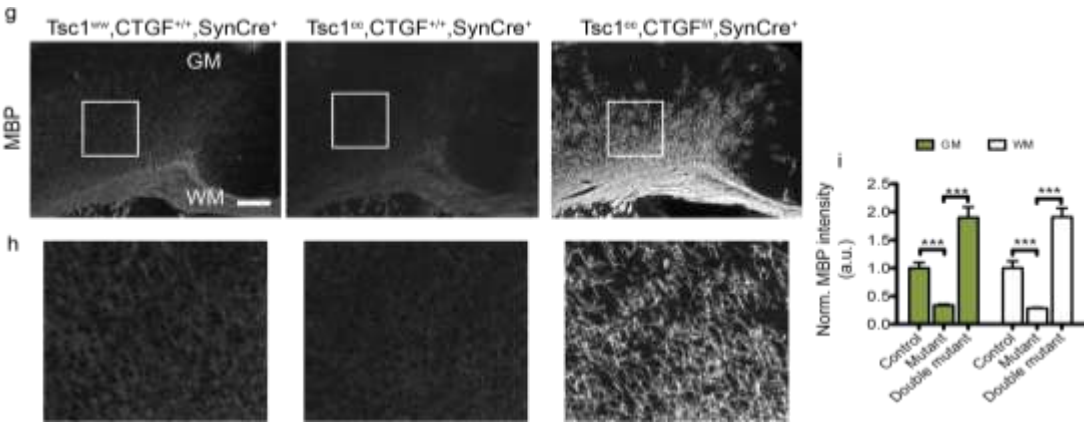

Figure 1: CTGF promotes myelination i*n vivo*. We generated the following groups of mice: wild type for both genes (Control: *Tsc1*^{ww}*Ctgf*^{ww}*SynICre*$^{+}$), mutant for *Tsc1* and wild type for *Ctgf* (*Tsc1* mutant: *Tsc1*^{cc}*Ctgf*^{ww}*SynICre*$^{+}$), mutant for both genes (double mutant: *Tsc1*^{cc}*Ctgf*^{cc}*SynICre*$^{+}$). g) We compared g) MBP staining of brain sections from control (n=5), *Tsc1* mutant (n=6) and double mutant (n=4) mice and h) Enlarged images corresponding to the area depicted with white square in (g). i) MBP signal quantifications of corresponding mice for GM WM in arbitrary units (a.u.).

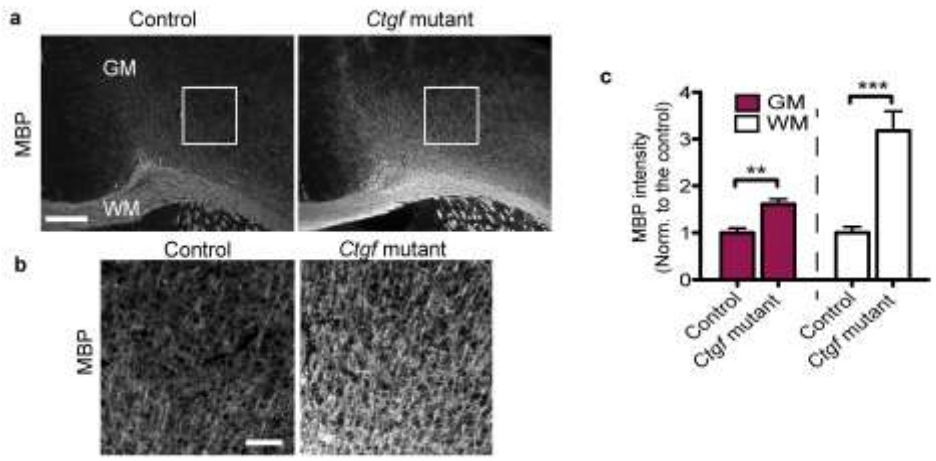

Figure 2: Loss of neuronal CTGF improves myelination. a) Representative images of MBP staining of the brain sections from the control (n=3) and *Ctgf* mutant (*Tsc1*^{ww}*SynICre*$^{+}$*Ctgf*^{cc}, n=3). Gray matter (GM) and the white matter (WM) and midline (M) regions are depicted. Scale bar corresponds to 500□m. b) Enlarged images from the depicted white squares in (a), scale bar corresponds to 150□m. c) Quantifications of MBP intensity in arbitrary units (a.u).

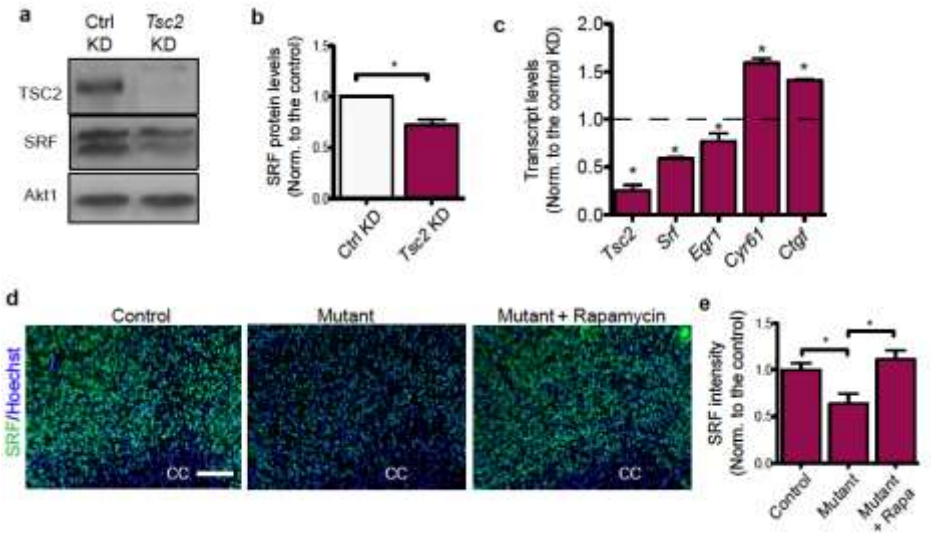

Figure 3. SRF is downregulated in *Tsc*-deficient neurons. a) Immunoblotting of control and *Tsc2* KD cortical neurons showing Tsc2, SRF and Akt1 (loading control). b) Quantification of the SRF protein levels normalized to Akt1 (n=3). c) qRT-PCR of *Tsc2, Srf, Egr1, Cyr61* and *Ctgf* in *Tsc2* KD primary cortical neurons (n=3), showing the transcript levels. The dashed line represents the transcript levels in the control KD, which is set to 1. d) SRF staining of brain sections from control (n=3), *Tsc1* mutant (n=3) and rapamycin treated mutant (n=3)